Mapping protein-DNA interactions with DiMeLo-seq

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Abstract

We recently developed **Di**rected **Me**thylation with **Lo**ng-read **seq**uencing (DiMeLo-seq) to map protein-DNA interactions genome wide. DiMeLo-seq maps multiple interaction sites on single DNA molecules, profiles protein binding in the context of endogenous DNA methylation, and maps protein-DNA interactions in repetitive regions of the genome that are difficult to study with short-read methods. Adenines in the vicinity of a protein of interest are methylated in situ by tethering the Hia5 methyltransferase to an antibody using protein A. Protein-DNA interactions are then detected by direct readout of adenine methylation with long-read, single-molecule, DNA sequencing platforms such as Nanopore sequencing. Here, we present a detailed protocol and guidance for performing DiMeLo-seq. This protocol can be run on nuclei from fresh, lightly fixed, or frozen cells. The protocol requires 1 day for performing in situ targeted methylation, 1-5 days for library preparation depending on desired fragment length, and 1-3 days for Nanopore sequencing depending on desired sequencing depth. The protocol requires basic molecular biology skills and equipment, as well as access to a Nanopore sequencer. We also provide a Python package, *dimelo*, for analysis of DiMeLo-seq data.

Introduction

Common methods for mapping protein-DNA interactions rely on selective amplification and sequencing of short DNA fragments from regions bound by the protein of interest.[1–7] These short-read methods for profiling protein-DNA interactions are powerful and have been used to map the binding patterns of thousands of proteins in human cells.[8] However, because the measurement is on short, amplified fragments of DNA, these methods dissociate joint binding information at neighboring sites, remove endogenous DNA methylation, and are limited in detecting haplotype-specific interactions and interactions in repetitive regions. DiMeLo-seq addresses these limitations by recording protein binding through the deposition of targeted methyladenine (mA) marks that are read out with long-read, single-molecule sequencing (Figure 1).[9]

Development of the protocol

DiMeLo-seq is a method for profiling protein-DNA interactions that relies on in situ antibody-targeted DNA methylation followed by direct readout of methylation with single-molecule, native DNA sequencing. The method was inspired by the targeted methylation strategy used in DamID-seq[7] and builds from short-read techniques for mapping protein-DNA interactions (e.g. CUT&Tag,[5] CUT&RUN,[6] and pA-DamID[10]), as well as recent work that implements long-read sequencing and detection of exogenous methylation to profile chromatin accessibility (Fiber-seq,[11] SMAC-seq,[12] SAMOSA,[13] NanoNOMe[14], MeSMLR-seq[15]). Developing DiMeLo-seq required substantial optimization, with over 100 conditions tested.[9] These optimization experiments revealed critical components that improved efficiency including the following: (1) Hia5 performed significantly better than EcoGII in situ; (2) compared to other detergents for nuclear permeabilization, digitonin and Tween-20 dramatically increased methylation levels; and (3) a low salt concentration, including BSA, increasing incubation time, and replenishing the methyl donor during activation all improved methylation levels.

Applications of the method

DiMeLo-seq can be used to profile the genome localization of any DNA-binding protein for which there is a specific, high-quality antibody. The protocol can be used on fresh, fixed, or frozen cells from culture or from primary tissue. Because DiMeLo-seq uses antibody-based targeting, DiMeLo-seq is also able to profile post-translational modifications like protein acetylation, methylation, and phosphorylation. In our previous work,[9] we demonstrated application of DiMeLo-seq for targeting LMNB1, CTCF, H3K9me3, and CENPA in cultured human cells, and here we applied DiMeLo-seq to profile H3K27ac, H3K27me3, and H3K4me3 in cultured human cells and H3K9me3 in *D. melanogaster* embryos.

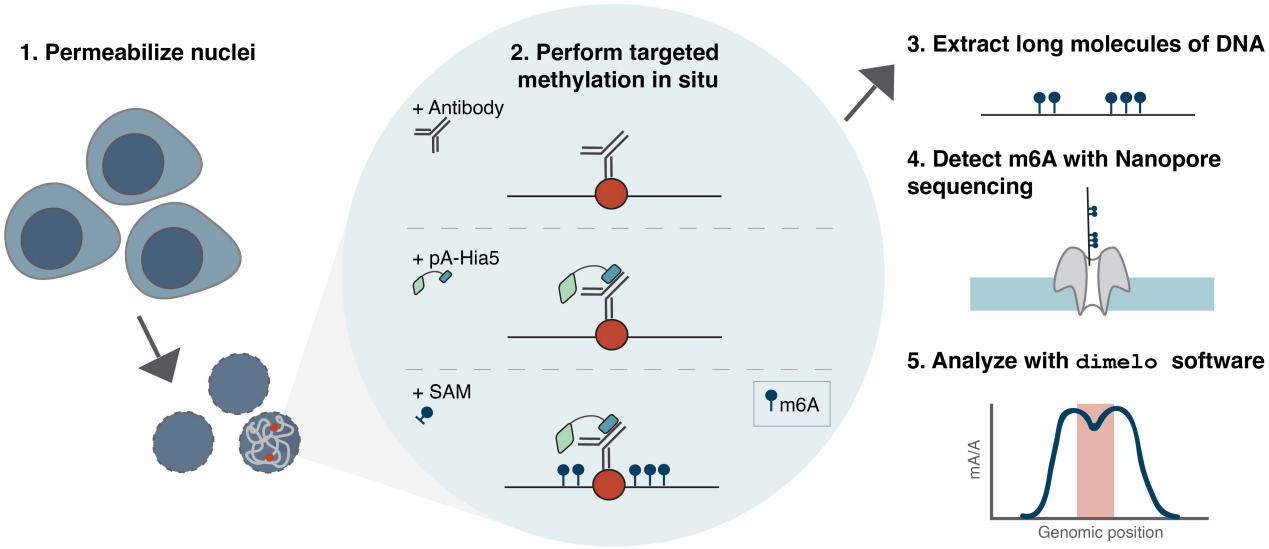


Figure 1. DiMeLo-seq protocol overview. 1. Permeabilize nuclei from fresh, frozen, or fixed cells. 2. Perform a series of steps within the permeabilized nuclei: (i) bind primary antibody to the protein of interest, (ii) bind pA-Hia5 to the primary antibody, (iii) add S-adenosylmethionine (SAM), the methyl donor, to activate methylation. 3. Extract long molecules of DNA. 4. Sequence this DNA with a Nanopore sequencer to detect m6A directly. 5. Analyze modified basecalls from sequencing using the *dimelo* software package.

Comparison with other methods

The key distinguishing feature of DiMeLo-seq compared to other methods is that protein-DNA interactions are measured on native, long molecules of DNA. Native DNA molecules allow for binding assessment in the context of endogenous CpG methylation. Long reads facilitate interaction mapping in highly repetitive regions of the genome, measurement of multiple binding events on the same chromatin fiber, and haplotype-specific interaction detection.

ChIP-seq, CUT&RUN, CUT&Tag, and DamID-seq all rely on amplification of short sequencing reads. These methods use coverage as a proxy for binding, and resolution is determined by the size of the fragments sequenced. With DiMeLo-seq, the resolution is tied to adenine density and the reach of the methyltransferase. Short reads often preclude haplotype phasing, mapping to repetitive regions, and measuring coordinated binding. These short-read methods require amplification, thereby losing the endogenous mCpG marks. Joint protein binding and mCpG measurement can be done with BisChIP-Seq/ChIP-BS-Seq, but this requires lossy and harsh bisulfite conversion that degrades DNA.[16,17] Similar to ChIP-seq, CUT&Tag, and CUT&RUN, DiMeLo-seq is compatible with primary cells and can be used to target post-translational modifications.

DiMeLo-seq requires substantial input to generate sufficient material for sequencing because there is no amplification, so typical experiments have ~1M cells as input. With short-read methods such as CUT&Tag and DamID-seq, protein-bound regions are selectively amplified and sequenced, thereby enriching for protein-bound regions in the final library for sequencing and allowing for input as low as a single cell. Without enrichment for regions of interest, DiMeLo-seq will sequence the whole genome uniformly, requiring deep sequencing to achieve sufficient coverage of specific target regions. However, there are options to enrich for regions of interest with DiMeLo-seq like AlphaHOR-RES,[9] the Oxford Nanopore Technologies Cas9-based targeted library preparation kit (SQK-CS9109), or m6A-IP as in MadID.[18]

Experimental design

While the standard DiMeLo-seq protocol described here has performed consistently for all cell types tested, application to other cell types and primary tissue may require tuning of digitonin concentration. A digitonin concentration of 0.02% has worked well for human GM12878, HG002, Hap1, HEK293T, and Drosophila S2. The optimal digitonin concentration can be determined using Trypan Blue (Figure 2a). Primary tissue also requires upstream processing for nuclear extraction before the nuclear permeabilization step (Supplementary Methods). It is important that the nuclear extraction method does not contain NP-40, as we have found this detergent can significantly reduce methylation.

Key variables to optimize for a new target protein are the antibody concentration and the extent of fixation for targets with low binding affinity. An antibody dilution of 1:50 has worked well for all targets reported here and in Altemose et al.[9] Extensive washes are performed after antibody binding to remove any unbound antibody, making excess antibody less detrimental. We have demonstrated that light fixation is compatible with the DiMeLo-seq workflow. If targeting a protein that binds transiently, including fixation may improve signal by preventing the protein from dissociating from the DNA during the DiMeLo-seq protocol.

Typical controls include an IgG isotype control and a free pA-Hia5 control. The IgG isotype control measures nonspecific antibody binding. The free pA-Hia5 control measures chromatin accessibility, similar to Fiber-seq and related methods, and is analogous to the Dam only control used in DamID-seq.[11,19] This control is performed by excluding the primary antibody and pA-Hia5 binding steps and instead adding pA-Hia5 at activation at 200 nM. While these controls are not required, they provide a useful measure of background methylation and bias caused by variable chromatin accessibility. Excluding pA-Hia5 as a control to account for modified basecalling errors can also be included. If troubleshooting a DiMeLo-seq experiment, using one of the antibodies and cell lines validated here and in Altemose et al.[9] may also be a useful control.

Commercially available kits and techniques for DNA extraction, library preparation, and sequencing are rapidly improving. The protocol described here produces consistent localization profiles shown below and in Altemose et al.,[9] but it is important to note that after the in situ methylation steps, any DNA extraction method, library preparation kit, flow cell chemistry, and sequencing device can be used as long as m6A is maintained (no amplification is performed) and the flow cell and device have basecalling models available for calling m6A. We have also demonstrated sequencing of DiMeLo-seq samples with Pacific Biosciences's Sequel IIe.[9]

The key considerations for sequencing are fragment size and sequencing depth. The target N50 (half of sequenced bases are from a fragment size of N50 or larger) varies by application. Longer reads may be desired when mapping to repetitive regions, probing coordinated binding events at longer distances, or phasing reads. With ligation-based library preparation we typically target an N50 of ~20 kb to ~50 kb, which results in fragment size distributions as in Figure 2b. With other library preparation kits (e.g., SQK-ULK001), much larger fragments can be sequenced; however, there is a tradeoff between fragment length and throughput. The target sequencing depth also varies by application and will depend on the binding footprint of the protein, the mappability of the region of interest, and the biological question at hand. Final libraries can be saved and flow cells can be reloaded, so it is recommended to do an initial pilot run with shallow sequencing followed by deeper sequencing as needed. For example, for initial tests of new protein targets in human cells, we typically sequence to ~1-3 Gb, or 0.3-1X coverage, to validate and determine optimal experimental conditions, and then sequence more deeply to ~5-45X coverage depending on the analysis we are performing. See sequencing the saturation analysis with CTCF-targeted DiMeLo-seq in Altemose et al.[9]

Expertise needed to implement the protocol

To perform DiMeLo-seq and analyze the data produced, basic molecular biology skills and basic command line skills are required.

Limitations

The performance of DiMeLo-seq is strongly dependent on the quality of the antibody used to target the protein of interest. For proteins that do not have a specific, high-quality antibody compatible with protein A, one could consider epitope tagging or performing in vivo expression of a protein-MTase fusion.[20] DNA must be accessible for Hia5 to methylate in situ. Thus, targets in less accessible regions of the genome may require longer incubations or deeper sequencing. Hia5 may methylate DNA in trans if close enough to the target protein in 3D space. DiMeLo-seq experiments typically require ~1M cells as input, although Concanavalin A beads (which we previously showed are compatible with DiMeLo-seq) and lower-input library preparation kits can reduce required input material.[9]

Here and in our previous study, we have benchmarked DiMeLo-seq's performance in targeting LMNB1, CTCF, H3K9me3, CENPA, H3K27ac, H3K27me3, and H3K4me3.[9] When targeting CTCF and LMNB1, we estimated 54% and 59% sensitivity (94% specificity), but this is dependent on the protein, antibody, and chromatin environment and must be evaluated for new targets.[9] Transiently bound proteins may benefit from the optional fixation step at the start of the DiMeLo-seq protocol.

Materials

REAGENTS

A. Reagents for in situ protocol

- HEPES-KOH 1 Molarity (M) pH 7.5 (Boston BioProducts BBH-75-K)
- NaCl 5 M (Sigma-Aldrich 59222C-500ML)
- Spermidine 6.4 M (Sigma-Aldrich S0266-5G)
- Roche cOmplete™ EDTA-free Protease Inhibitor Tablet (Sigma-Aldrich 11873580001)
- Bovine Serum Albumin (Sigma-Aldrich A6003-25G)
- Digitonin (Sigma-Aldrich 300410-250MG)
  CAUTION acute toxic and health hazard; work in fume hood when making digitonin solution.
- Tween-20 (Sigma-Aldrich P7949-100ML)
- Tris-HCl 1M pH 8.0 (Invitrogen 15568025)
- KCl (Sigma-Aldrich PX1405-1)
- EDTA 0.5 M pH 8.0 (Invitrogen 15575-038)
- EGTA 0.5 M pH 8.0 (Fisher 50-255-956)
- S-Adenosylmethionine 32 mM (NEB B9003S)
- PFA, 16% (if performing fixation) (EMS 15710)
- Glycine (if performing fixation) (Fisher BP381-1)
- Eppendorf DNA LoBind tubes 1.5 mL (Fisher 022431021)
- Wide bore 200 µl and 1000 µl tips (e.g. USA Scientific 1011-8810, VWR 89049-168)
- pA-Hia5 (see https://www.protocols.io/view/pa-hia5-protein-expression-and-purification-x54v9j56mg3e/v1 for expression and purification protocol. The pET-pA–Hia5 (pA-Hia5) plasmid is available from Addgene (cat no. 174372)).
- Primary antibody for protein target of interest, from species compatible with pA (e.g. Abcam ab16048)
- Secondary antibody for immunofluorescence quality control (e.g. Abcam ab3554)
- Trypan Blue (Fisher T10282)
- Qubit dsDNA BR Assay Kit (Fisher Q32850)
- Qubit Protein Assay Kit (Fisher Q33211)

B. Reagents for extraction, library preparation, and sequencing

N.B. We have validated the following reagents, but extraction, library preparation, and sequencing reagents are improving rapidly. The important considerations are to choose a DNA extraction method that maintains long DNA molecules, to perform amplification-free library preparation, and to use a flow cell that is compatible with mA calling.

- Monarch Genomic DNA Purification Kit (NEB T3010S)
- Monarch HMW DNA Extraction Kit (NEB T3050L)
- Agencourt AMPure XP beads (Beckman Coulter A63881)
- Blunt/TA Ligase Master Mix (NEB M0367S)
- NEBNext quick ligation module (NEB E6056S)
- NEBNext End Repair dA-tailing Module (NEB E7546S)
- NEBNext FFPE DNA repair kit (NEB M6630S)
- Ligation Sequencing Kit (ON SQK-LSK109, ON SQK-LSK110, or latest kit compatible with m6A calling)
- Native Barcoding Expansion 1-12 (ON EXP-NBD104, or latest kit compatible with m6A calling)
- Native Barcoding Expansion 13-24 (ON EXP-NBD114, or latest kit compatible with m6A calling)
- Circulomics Short Read Eliminator Kit (SS-100-101-01)
- Flow Cell Wash Kit (ON EXP-WSH004)
- Flow cells (ON FLO-MIN106D or ON FLO-PRO002, or latest flow cells compatible with m6A calling)

EQUIPMENT

- Centrifuge that can hold 4ºC
- Rotator (e.g. Millipore Sigma Z740289)
- Oxford Nanopore Technologies Nanopore sequencer (e.g. MIN-101B)
- Magnetic separation rack (if targeting N50 ~20 kb) (e.g. NEB S1515S)
- Qubit (e.g. Thermo Fisher Scientific Q33238)
- Tapestation (not required; for quality control) (e.g. Agilent G2992AA)
- Microscope (not required; for quality control)

REAGENT SETUP

A. Buffer preparation

Prepare all buffers fresh, filter buffers through a 0.2 µm filter, and keep buffers on ice.

Digitonin

Solubilize digitonin in preheated 95ºC Milli-Q water to create a 5% digitonin solution (e.g. 10 mg/200 µl).

Wash Buffer

Prepare wash buffer according to the following table.

Component	Amount	Final Concentration
HEPES-KOH, 1 M, pH 7.5	1 ml	20 mM
NaCl, 5 M	1.5 ml	150 mM
Spermidine, 6.4 M	3.91 µl	0.5 mM
Roche Complete tablet -EDTA	1 tablet	-
BSA	50 mg	0.1%
H2O	up to 50 ml	-

Dig-Wash Buffer

Add 0.02% digitonin to wash buffer. For example, add 20 µl of 5% digitonin solution to 5 ml wash buffer. The optimal concentration of digitonin may vary by cell type.

Tween-Wash Buffer

Add 0.1% Tween-20 to wash buffer. For example, add 50 µl Tween-20 to 50 ml wash buffer.

Activation Buffer

Prepare the activation buffer but wait to add SAM until the activation step.

Component	Amount	Concentration
Tris, pH 8.0 1 M	750 µL	15 mM
NaCl 5 M	150 µL	15 mM
KCl 1 M	3 mL	60 mM
EDTA, pH 8.0 0.5 M	100 µL	1 mM
EGTA, pH 8.0 0.5 M	50 µL	0.5 mM
Spermidine, 6.4 M	0.391 µL*	0.05 mM

BSA	50 mg	0.1%
H2O	up to 50 mL	-
SAM, 32 mM	(add at activation step)	800 µM

*To reduce pipetting error, first perform a 1:10 dilution of Spermidine in H2O by adding 1µLof 6.4 M Spermidine to 9 µL H2O. Mix well and then add 3.91 µL of this dilution to the Activation Buffer.

Procedure

General notes

- The protocol will be kept up-to-date at: https://www.protocols.io/view/dimelo-seq-directed-methylation-with-long-read-seq-n2bvjxe4wlk5/v2
- All spins are at 4°C for 3 minutes at 500 g.
- Spinning in swinging bucket rotor can help pellet the nuclei.
- To prevent nuclei from lining the side of the tube, break all spins into two parts: 2 minutes with the tube hinge facing inward, followed by 1 minute with the tube hinge facing outward. This two-part spin is not needed if using a swinging bucket rotor.
- Working with Eppendorf DNA LoBind tubes can reduce loss of material.
- Use wide bore tips when working with nuclei.
- Do not use NP-40 or Triton-X100 for nuclear extraction, permeabilization, or any other stage of the protocol, as they appear to dramatically reduce methylation activity.
- The best digitonin concentration may vary by cell type. For HEK293T, GM12878, HG002, Hap1, and S2 cells, 0.02% works well. You can test different concentrations of digitonin and verify permeabilization and nuclear integrity by Trypan blue staining. For example, you may try 0.02% to 0.1% digitonin.
- We use Tween to reduce hydrophilic non-specific interactions and BSA to reduce hydrophobic non-specific interactions. We also found that including BSA at the activation step significantly increases methylation activity as well.
- The best primary antibody concentration may vary by protein target of interest. A 1:50 dilution works well for targeting LMNB1, CTCF, and histone modifications, and is likely a good starting point for most antibodies.
- Binding a secondary antibody after the primary antibody but before pA-Hia5 reduced total methylation and specificity. Including a secondary antibody binding step is not recommended. For pA-incompatible antibodies, a secondary antibody can be used as a bridging antibody, but performance is diminished; instead, we recommend using pAG-Hia5 for pA-incompatible antibodies.

<u>(Optional fixation)</u>

TIMING 10 minutes

1. Resuspend cells in PBS (1 million to 5 million cells per condition).
2. Add PFA to 0.1% (e.g. 6.2 µl of 16% PFA to 1 ml cells) for 2 minutes while gently vortexing.
3. Add 1.25 M glycine (sterile; 0.938 g in 10 ml) to twice the molar concentration of PFA to stop the crosslinking (e.g. 60 µl of 1.25 M glycine to 1 ml).
4. Centrifuge 3 minutes at 500 x g at 4°C and remove the supernatant.
5. Continue with Nuclear Isolation, starting with step 8.

Nuclear isolation

TIMING 15 minutes

6. Prepare cells (1M-5M per condition).
7. Wash cells in PBS. Spin and remove supernatant.
8. Resuspend cells in 1 ml Dig-Wash buffer. Incubate for 5 minutes on ice.
   TROUBLESHOOTING
9. Split nuclei suspension into separate tubes for each condition.
10. Spin and remove supernatant.
11. Quality control: Check permeabilization was successful by taking 1 µl of the nuclei following the 5-minute incubation on ice, diluting to 10 µl with PBS, and staining with Trypan Blue.

Primary antibody binding

TIMING 2.5 hours

12. Gently resolve each pellet in 200 µl Tween-Wash containing primary antibody at 1:50 or the optimal dilution for your antibody and target.
13. Place on rotator at 4°C for ~2 hr.
    PAUSE POINT - Samples can be left overnight on the rotator at 4°C.
14. Spin and remove supernatant.
15. Wash twice with 0.95 ml Tween-Wash. For each wash, gently and completely resolve the pellet. This may take pipetting up and down ~10 times. Following resuspension, place on rotator at 4°C for 5 minutes before spinning down.

Quantify pA-Hia5 concentration

TIMING 30 minutes

16. Thaw protein from -80°C at room temperature and then move to ice immediately.
17. Spin at 4°C for 10 minutes at 10,000 x g or higher to remove aggregates.
18. Transfer the supernatant to a new tube and save it, discarding the previous tube.
19. Use Qubit with 2 µl sample volume to quantify protein concentration.

pA-Hia5 binding

TIMING 2.5 hours

20. Gently resolve pellet in 200 µl Tween-Wash containing 200 nM pA-Hia5.
21. Place on rotator at 4°C for ~2 hr.
22. Spin and remove supernatant.
23. Wash twice with 0.95 ml Tween-Wash. For each wash, gently and completely resolve the pellet. Following resuspension, place on rotator at 4°C for 5 minutes before spinning down.

Quality control (optional)

TIMING 1 hour

24. Add 1.6 µl of 16% PFA to 25 µl of nuclei in Tween-Wash (taken from the 0.95 ml final wash) for 1% total PFA concentration.
25. Incubate at room temperature for 5 minutes.
26. Add 975 µl of Tween-Wash to stop the fixation by dilution.
27. Add 1 µl fluorophore-conjugated secondary antibody.
28. Put on rotator for 30 minutes at room temperature, protected from light.
29. Wash 2 times (or just once). Pellet likely won't be visible.
30. Resuspend in mounting media after last wash. Use as little as possible, ideally 5 µl.
31. Put 5 µl on a slide, make sure there are no bubbles, and put on a coverslip.
32. Seal with nail polish along the edges.
33. Image or put at -20°C once the nail polish has dried.
    TROUBLESHOOTING

Activation

TIMING 2.5 hours

34. Gently resolve pellet in 100 µl of Activation Buffer per sample. Be sure to add SAM to a final concentration of 800 µM in the activation buffer at this step! In 100 µl of Activation Buffer, this means adding 2.5 µl of the SAM stock that is at 32 mM.
35. Incubate at 37°C for 2 hours. Replenish SAM by adding an additional 800 µM at 1 hour. This means adding an additional 2.5 µl of the SAM stock that is at 32 mM to the 100 µl reaction. Pipet mix every 30 minutes. Tapping to mix also works.
36. Spin and remove supernatant.
37. Resuspend in 100 µl cold PBS.
38. Check nuclei by Trypan blue staining to determine recovery and check integrity of nuclei if desired.

Depending on desired fragment size, either follow Method A for N50 ~20 kb or Method B for N50 ~50 kb.

N.B. We have validated the following reagents, but extraction, library preparation, and sequencing reagents are improving rapidly. The important considerations are to choose a DNA extraction method that maintains long DNA molecules, to perform amplification-free library

preparation, and to use a flow cell that is compatible with mA calling. These are workflows we have validated and modifications we have made.

A. DNA extraction for N50 of ~20 kb

TIMING 1 hour

39. Use the Monarch Genomic DNA Purification Kit. Follow protocol for genomic DNA isolation using cell lysis buffer. Include RNase A. NB. If fixation was performed, be sure to do the 56°C incubation for lysis for 1 hour (not just 5 minutes) to reverse crosslinks.
40. Perform two elutions: 100 µl and then 35µl.
    PAUSE POINT - Samples can be stored at 4°C or -20°C.
41. Quantify DNA yield by Qubit dsDNA BR Assay Kit.
42. Concentrate by speedvac if necessary for 1-3 µg DNA in 48 µl for input to library prep.

B. DNA extraction for N50 ~50 kb

TIMING 1 hour

43. Use the NEB Monarch HMW DNA Extraction Kit. Follow protocol for genomic DNA isolation using cell lysis buffer. Include RNase A. Perform lysis with 2000 rpm agitation. We have validated 2000 rpm gives N50 ~50-70 kb but if longer reads are desired we expect 300 rpm would work. Apart from using a different kit, all of the steps for the long fragment DNA extraction are the same as the general protocol. To reiterate, make the following changes to the protocol outlined in the following steps. If fixation was performed, be sure to do the 56°C incubation for lysis for 1 hour (not just 10 minutes) to reverse crosslinks. Agitate for 10 minutes and then keep at 56ºC without agitation for 50 minutes.
    PAUSE POINT - Samples can be stored at 4°C or -20°C.
44. Quantify DNA yield by Qubit dsDNA BR Assay Kit.
45. Concentrate by speedvac if necessary to obtain 1-3 µg DNA in 48 µl for input to library prep.

(Optional enrichment)

46. If the sequencing cost and time for sufficient coverage becomes prohibitive, a few enrichment strategies can be used. A restriction enzyme-based approach like AlphaHOR-RES relies on preferential digestion of DNA outside of target regions followed by size selection to maintain larger on-target fragments.[9] The ONT Cas9 Sequencing Kit (SQC-CS9109) is another option to selectively ligate adapters to targeted regions during library preparation. To enrich for methylated regions, m6A-specific immunoprecipitation can be used, as in MadID.[18]

A. Library preparation & sequencing for N50 ~20 kb

TIMING 3 hours

47. If multiplexing samples on a flow cell, follow Nanopore protocol for Native Barcoding Ligation Kit 1-12 and Native Barcoding Ligation Kit 13-24 with ON SQK-LSK109. If not multiplexing, use ON SQK-LSK110. We recommend the following modifications:
    a. Load ~3 µg DNA into end repair.
    b. Incubate for 10 minutes at 20°C for end repair instead of 5 minutes.
    c. Load ~ 1 µg of end repaired DNA into barcode ligation.
    d. Double the ligation incubation time(s) to at least 20 minutes.
    e. Elute in 18 µl instead of 26 µl following barcode ligation reaction cleanup to allow for more material to be loaded into the final ligation.
    f. Load ~3 µg of pooled barcoded material into the final ligation. If needed, concentrate using speedvac to be able to load 3 µg into the final ligation.
    g. Perform final elution in 13 µl EB. Take out 1 µl to dilute 1:5 for quantification by Qubit (and size distribution analysis by TapeStation / Bioanalyzer if desired).
    h. Load ~1 µg of DNA onto the sequencer. Input requirements vary by sequencing kit and are becoming lower.

    TROUBLESHOOTING

B. Library preparation & sequencing for N50 ~50 kb

TIMING 5 days

48. Follow Nanopore protocol for ON SQK-LSK110 (method validated with this kit only, not with multiplexing with ON SQK-LSK109) with the following modifications (inspired by Kim et al, dx.doi.org/10.17504/protocols.io.bdfqi3mw):[21]
    a. Increase end preparation time to 1 hour with a 30-minute deactivation.
    b. Following end preparation, perform a cleanup by combining 60 µL SRE buffer from Circulomics (SS-100-101-01) with the 60 µL end prep reaction.
    c. Centrifuge this reaction at 10,000 x g at room temperature for 30 minutes (or until DNA has pelleted).
    d. Wash pelleted DNA with 150 µL of 70% ethanol two times, using a 2 minute spin at 10,000 x g between washes.
    e. Resuspend the pellet in 31 µL EB.
    f. Incubate at 50ºC for 1 hour. Incubate at 4ºC for at least 48 hours.
    g. For the ligation step, reduce ligation volume by half (total of 30 µL DNA in a 50 µL reaction volume). Increase the ligation incubation to 1 hour.
    h. Pellet DNA at 10,000 x g at room temperature for 30 minutes.
    i. Wash the pellet twice with 100 µL LFB, using a 2 minute spin at 10,000 x g between washes.
    j. Resuspend the pellet in 31 µL EB.
    k. Incubate at least 48 hours at 4ºC.
    l. Load 500 ng of DNA onto the sequencer. Input requirements vary by sequencing kit and are becoming lower.

m. If you see the number of active pores has dropped considerably after 24 hours, you can recover pore activity using the flow cell wash kit, then loading additional library material.

TROUBLESHOOTING

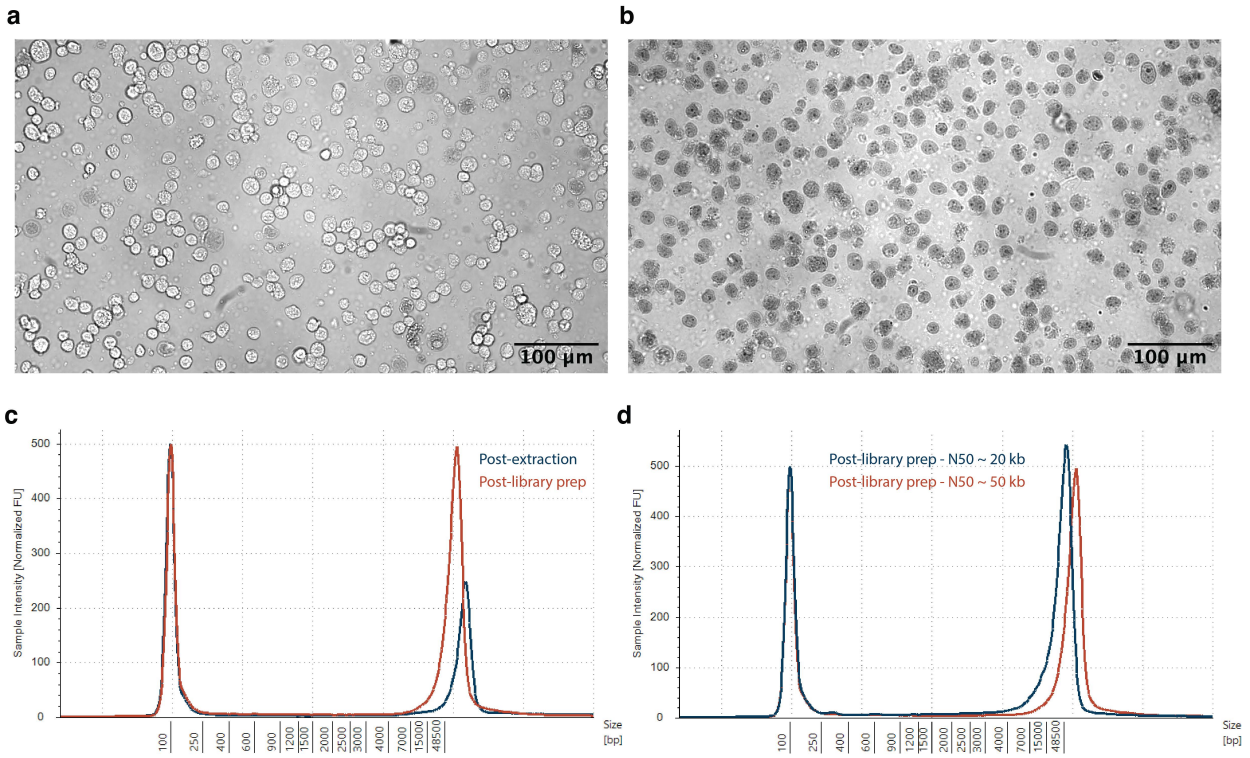


Figure 2. Experimental quality control. **a-b,** To determine successful permeabilization, cells are stained with Trypan Blue before (a) and after (b) digitonin treatment. Successful permeabilization allows Trypan Blue to enter the nuclei, while still maintaining high recovery of nuclei from cells. Over-permeabilization results in lower recovery of nuclei. Under-permeabilization does not allow Trypan Blue to enter the nuclei. **c-d,** After the DiMeLo-seq in situ protocol and DNA extraction, DNA is sized using the TapeStation. Representative traces from ligation-based library preparation are show in (c) for the fragment size distribution after extraction and after library preparation. In (d), the size distribution after library preparation for the two ligation-based methods presented in this protocol are shown. The blue curve results in N50 ~20 kb, while the red curve results in N50 ~50 kb. Larger fragment sizes can be achieved with other ultra long kits.

Analysis

After sequencing, the raw output files produced by the Nanopore sequencer must be converted to BAM files for input to a Python package called *dimelo* that we have created for analysis of DiMeLo-seq data (https://github.com/streetslab/dimelo). Recommendations for the basecalling and alignment steps, which will create an aligned BAM file with "Mm" and "Ml" tags that describe methylation calls, can be found in the package documentation (https://streetslab.github.io/dimelo/). Basecalling is being rapidly improved by ONT and others, so basecalling suggestions are likely to become outdated quickly. After basecalling and alignment, the resulting BAM file is the input to the quality control, visualization, and custom analysis functions from the *dimelo* software package. This analysis software can either be run as an imported Python module or can be run from the command line. A summary of the functions is in Figure 3.

A recommended workflow is to first run `qc_report` to generate summary statistics and histograms for metrics such as coverage, read length, mapping quality, basecall quality, and alignment quality (Figure 4). Next, three functions are provided for visualization. All functions take BAM file(s) as input and region(s) of interest defined as a string or bed file.

The `plot_enrichment` function compares methylation levels across samples or across different genomic regions. This tool is useful for looking at overall on- vs. off-target methylation and for comparing methylation levels in regions of interest across samples.

The `plot_browser` function allows the user to view single molecules with base modifications colored according to the probability of methylation within a region of interest. This function can either produce a static PDF of the single molecules or an interactive HTML file that allows the user to zoom and pan around the browser plot, using plotting code adapted from De Coster et al.[22] Plots of aggregate coverage and the fraction of methylated bases over the window of interest are also generated with this function.

The `plot_enrichment_profile` function creates single-molecule plots and an aggregate plot of the fraction of methylated bases centered at features of interest defined in a bed file. For example, one may enter a bed file with the locations of the binding motif for a given protein or with transcription start site coordinates to view the methylation profiles around these features of interest. Inputting multiple BAM files creates an overlay of the methylation profiles across samples and inputting multiple bed files creates an overlay of methylation profiles for a given sample across the different sets of regions defined in the bed files.

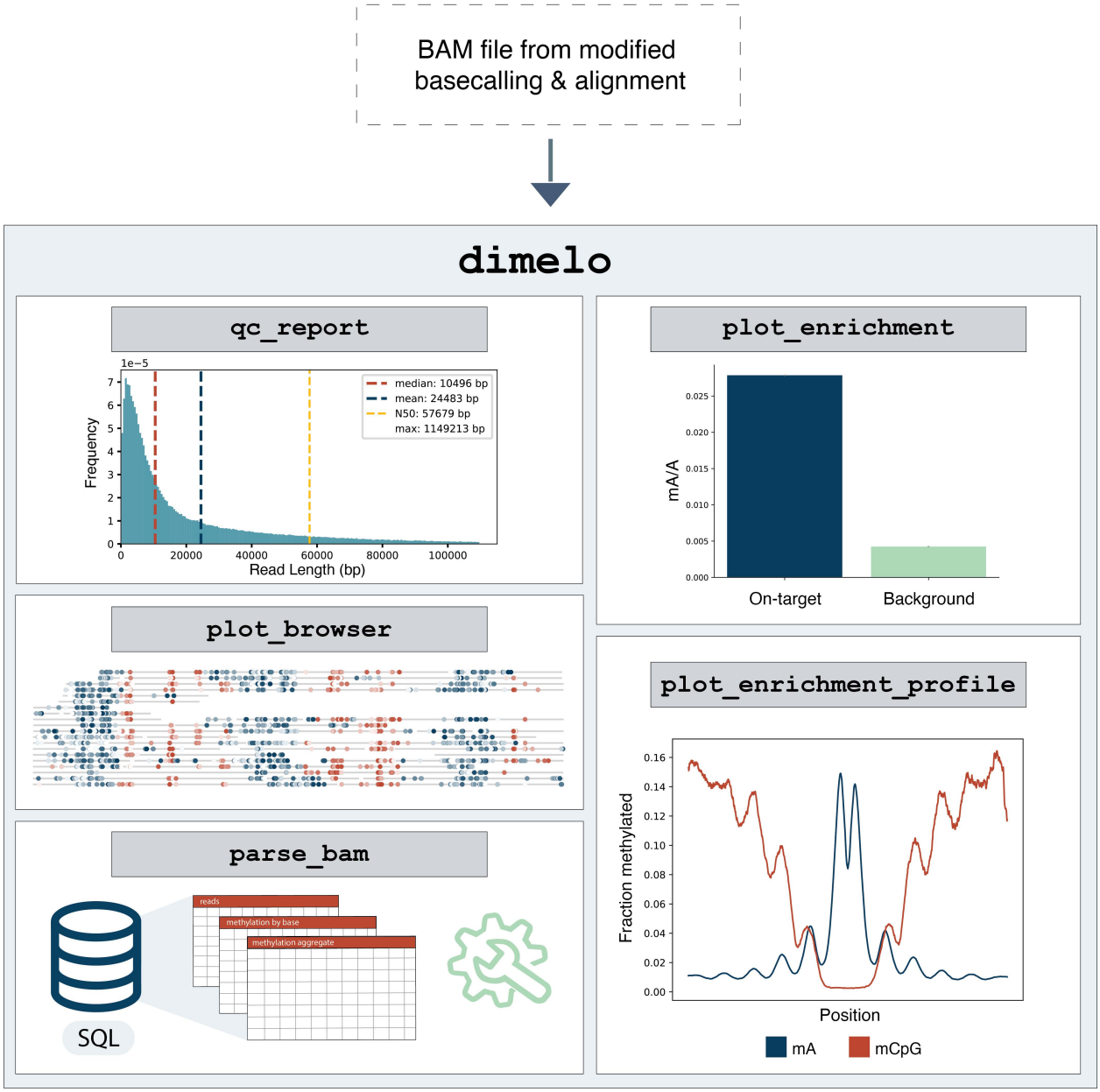
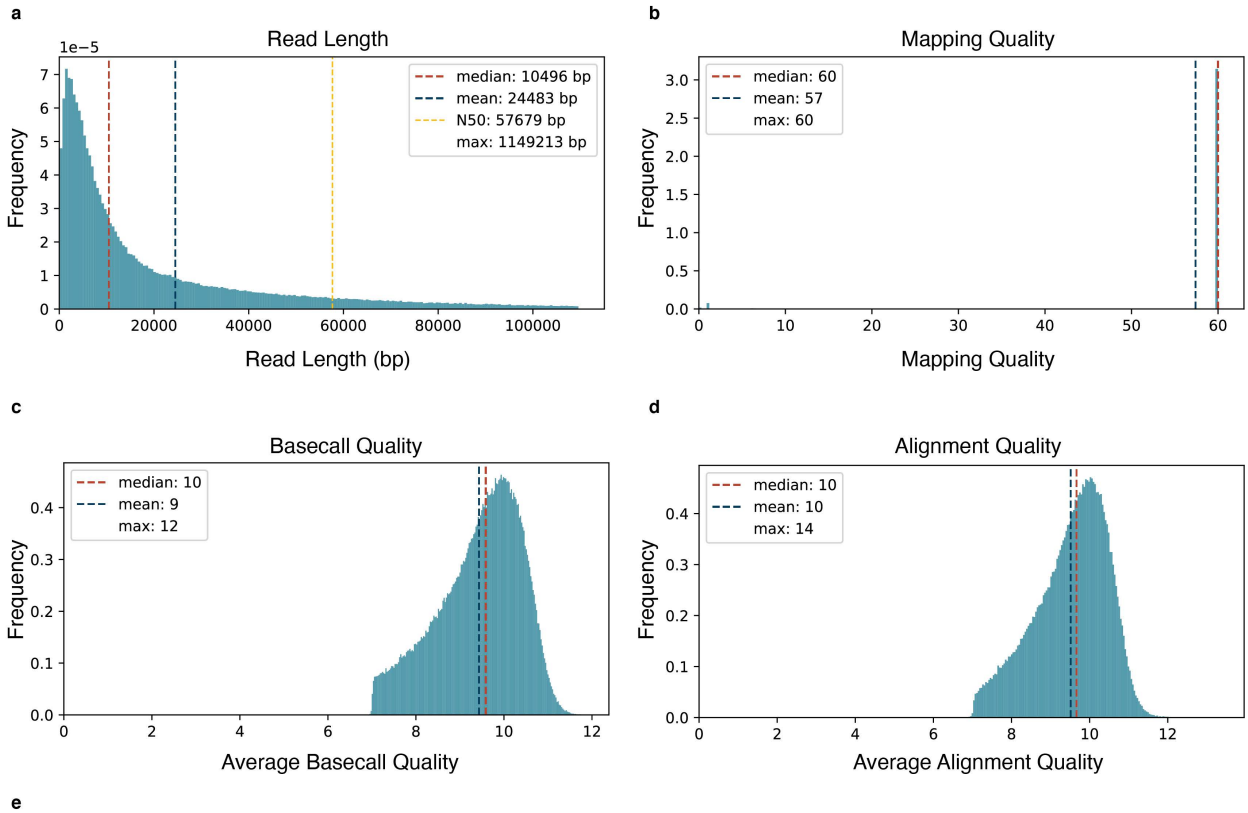


Figure 3. Analysis pipeline overview. Basecalling and alignment are performed on the fast5 output from the Nanopore sequencer. The resulting bam that contains the modified base information is then input to the *dimelo* software package. A recommended workflow involves quality control with `qc_report` followed by visualization with `plot_browser`, `plot_enrichment`, and `plot_enrichment_profile`. For custom analysis, `parse_bam` creates a SQL database with base modification calls in a format that makes it easier to manipulate for downstream analysis.



e

mean length: 24483 bp; num reads: 347136; num bases: 146898

	Read Length	Mapping Quality	Basecall Quality	Alignment Quality
Min	137	0	7	6
25%	4214	60	9	9
Median	10496	60	10	10
75%	30566	60	10	10
Max	1149213	60	12	14
Mean	24483	57	9	10

Figure 4. Sequencing quality control. The `qc_report` function takes in one or more bam files and for each, outputs a QC report including the following 5 features. **a,** Histogram of read lengths with the median, mean, N50, and max value annotated. **b,** Histogram of mapping quality. **c-d,** Both the basecall quality and alignment quality scores are present in bam outputs from Guppy but not from Megalodon. **c,** Histogram of average basecall quality per read. Here, the mean indicates our sample's average basecall quality is Q10 which is equivalent to 90% accuracy. **d,** Histogram of average alignment quality per read. While mapping quality provides the accuracy of the read mapping to specific genomic coordinates, the average alignment quality provides the quality of matching between the read and the reference sequence. For example, if a read almost perfectly matches multiple genomic coordinates, it will have a low mapping quality but a high alignment quality. **e,** Summary table with descriptive statistics of each feature (a-d), in addition to highlighting important values such as mean length of reads, total number of reads, and total number of bases sequenced. Example data used in this figure are from targeting H3K9me3 in *D. melanogaster* embryos.

The `parse_bam` function converts the base modification information stored in the BAM file into a SQL database to give users the option to create custom figures or analysis with the data in an easier format to manipulate.

For all functions, the user can specify the modification(s) of interest to extract - "A", "CG", or "A+CG". The probability threshold for calling a base as modified is also a parameter to each function. For discussion of threshold determination see Supplementary Note 6 of Altemose et al.[9]

Timing

N50 ~20 kb
Day 0
Steps 1-38, perform in situ targeted methylation: 10 h
Steps 39-42, DNA extraction: 1 h (2 h if fixation was performed)

Day 1
Step 47, perform library preparation & start sequencing: 3 h

Day 2
Step 47, re-load sequencer if necessary: 1 h

Day 3
Step 47, re-load sequencer if necessary: 1 h

N50 ~50 kb
Day 0
Steps 1-38, perform in situ targeted methylation: 10 h
Steps 43-45, DNA extraction: 2 h (3 h if fixation was performed)

Day 1
Step 48, perform library preparation end repair and clean: 2 h

Day 3
Step 48, perform library preparation ligation and clean: 2 h

Day 5
Step 48, start sequencing

Day 6

Step 48, re-load sequencer if necessary: 1 h

Day 7
Step 48, re-load sequencer if necessary: 1 h

Troubleshooting
Troubleshooting advice can be found in Table 1.

Step	Problem	Possible Reason	Solution
8	Few intact nuclei	Digitonin concentration is not optimal for the cell type	Try a range of digitonin concentrations and perform QC with Trypan Blue stain
33	No difference in fluorescence between IgG control and targeted methylation	Target abundance is low and/or target is diffuse	IF may not be a good quality control step for your target
		Insufficient washing	Add another wash step after secondary antibody binding
		Antibody concentration is not optimal	Try a range of primary and secondary antibody concentrations
		Primary or secondary antibody is not working	Try different antibody
		Permeabilization failure	To confirm permeabilization, perform Trypan Blue quality control step with varying digitonin concentrations.
47, 48	Unable to pipette viscous DNA	DNA is too long	Fragment DNA or follow library preparation protocol for persevering longer fragments (Step 48)
47	Bead clumping	DNA is too long for bead-based cleanup	Fragment DNA or follow library preparation protocol for preserving longer fragments (Step 48)
47	Low recovery from bead cleanup	DNA is too long for bead-based cleanup	Fragment DNA or follow library preparation protocol for preserving longer fragments (Step 48)

		DNA is too short for long fragment buffer (LFB) used in bead cleanup.	Handle HMW DNA carefully with wide bore tips and ensure your DNA extraction method maintains long DNA fragments.
47, 48	Short reads	DNA sheared during library preparation	Handle HMW DNA carefully with wide bore tips; follow library preparation protocol for preserving longer fragments (Step 48)
		Too much DNA loaded onto sequencer	Repeat qubit of final library. For target N50 ~20 kb, load ~1 µg of library; for target N50 ~50 kb, load 300 - 500 ng of library.
47, 48	Low yield from sequencer	Low input and long DNA fragments cause pores to become inactive quickly	Perform flow cell wash and reload every ~24 hours and/or load more DNA onto the flow cell. Washing and reloading becomes very important with larger fragment sizes.
		Bubbles destroy pores.	Use a new flow cell and be sure not to introduce bubbles during the flow cell loading process.

Table 1. Troubleshooting tips.

Anticipated results

One person can collect and analyze sequencing data within 3-8 days of beginning the DiMeLo-seq protocol. In this section, we show representative data from DiMeLo-seq experiments targeting H3K27ac, H3K27me3, and H3K4me3 in GM12878 cells and H3K9me3 in *D. melanogaster* embryos (Table 2). We use these targets to provide example output from the *dimelo* package and include suggested figures to evaluate performance and to perform exploratory analysis with DiMeLo-seq data.

Target	Cell type	Antibody	Library prep kit	Flow cell chemistry	Device	Gb	Coverage	N50 (bp)
H3K27ac	GM12878	Active Motif 39133	SQK-LSK110	R9.4.1	PromethION	124	41X	25,536
H3K27me3		Active Motif 39055				122	41X	27,226
H3K4me3		Active Motif 39916				124	41X	25,163
H3K9me3	*D. melanogaster* embryo	Active Motif 39062			MinION	8.24	46X	27,843

Table 2: Experimental overview. Summary of experimental specifications for histone modifications profiled using DiMeLo-seq.

The specificity and efficiency of methylation vary by target, depending on the antibody quality, how broad the binding domain is, and the chromatin environment, among other factors. The on-target and off-target methylation levels when targeting H3K27ac, H3K27me3, and H3K4me3 with DiMeLo-seq are shown in Figure 5a-c. These plots are generated from the `plot_enrichment` function. For H3K27ac, to define on-target regions, we used top ChIP-seq peaks for H3K27ac (ENCODE ENCFF218QBO).[23] For off-target, we used top ChIP-seq peaks for H3K27me3 (ENODE ENCFF119CAV).[23] We similarly analyze on- and off-target for H3K27me3 with H3K27me3 top ChIP-seq peaks for on-target and H3K27ac top ChIP-seq peaks for off-target regions. For H3K4me3, to define on-target regions, we used top ChIP-seq peaks for H3K4me3 (ENCODE ENCFF228TWF);[23] for off-target, we used transcription start sites of unexpressed genes where H3K4me3 is not expected to be present. The on-target methylation levels are higher for H3K27me3 compared to H3K27ac, despite H3K27me3 being a repressive mark in a less accessible genomic context. This is likely because it binds a broader genomic region, allowing a larger methylated footprint. The performance difference could also occur if the anti-H3K27me3 antibody performs better than the anti-H3K27ac antibody used in these experiments. The off-target methylation level is also higher in H3K27me3 compared to H3K27ac. This is likely because the off-target region used in this analysis is H3K27ac ChIP-seq peaks, which are in very accessible regions of the genome, and off-target methylation with DiMeLo-seq occurs preferentially within open chromatin. Again, higher off-target methylation can also be caused by differences in antibody performance.

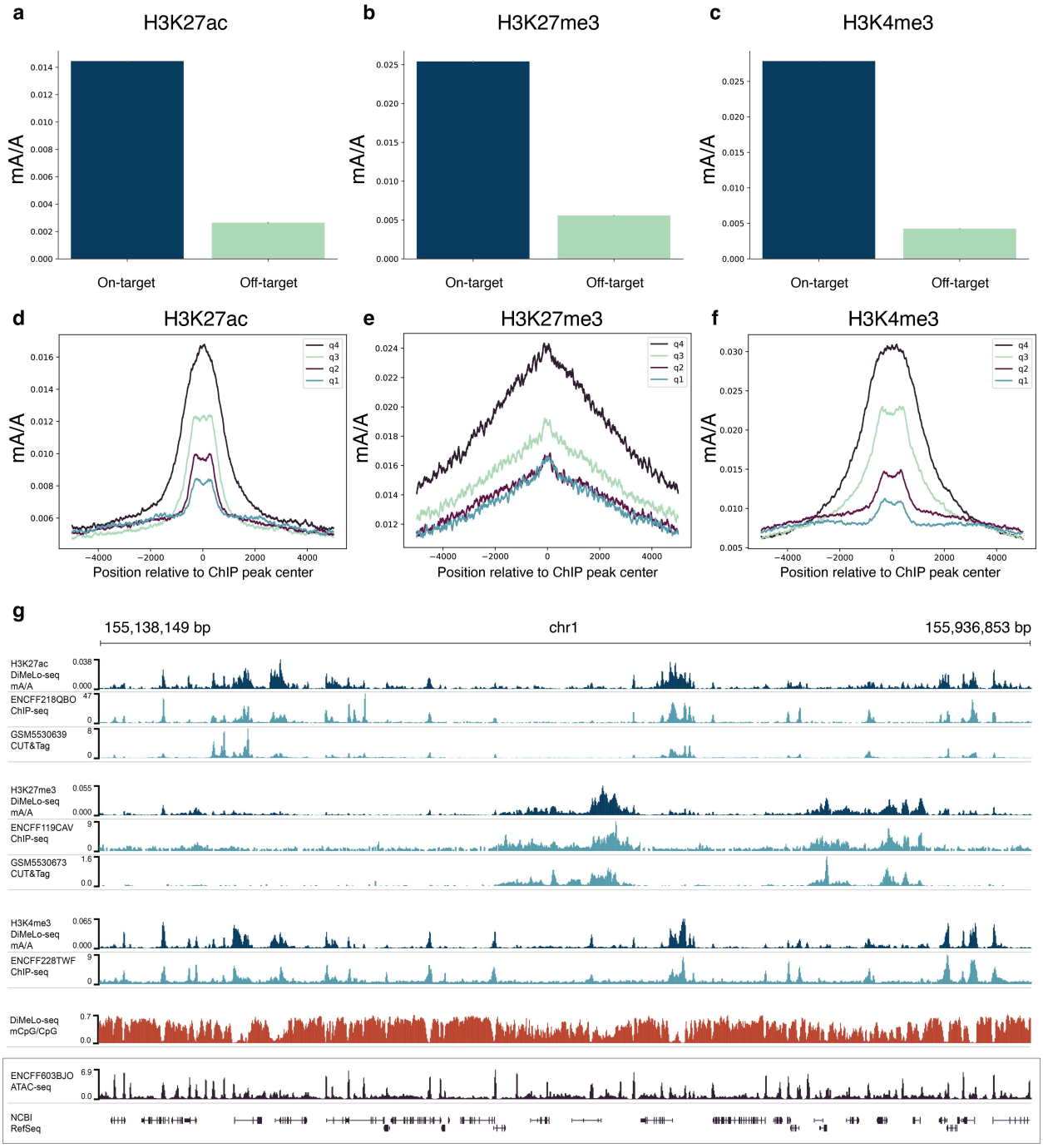


Figure 5. Validation of targeted methylation. **a-c,** Using bed files defining on- and off-target regions, the `plot_enrichment` function can be used to determine whether methylation is concentrated within expected regions. We've defined on-target regions using ChIP-seq peaks for the corresponding histone marks. We defined off-target regions when targeting H3K27ac as H3K27me3 ChIP-seq peaks and when targeting H3K27me3 as H3K27ac ChIP-seq peaks; for off-target regions for H3K4me3 we use transcription start sites for unexpressed genes. Methylation probability threshold of 0.75 was used. Error bars represent 95% credible intervals determined for

each ratio by sampling from posterior beta distributions computed with uninformative priors. **d-f,** Methylation profiles centered at ChIP-seq peaks for H3K27ac-, H3K27me3-, and H3K4me3-targeted DiMeLo-seq are plotted using `plot_enrichment_profile`. The quartiles (q4 to q1) indicate the strength of the ChIP-seq peaks which the DiMeLo-seq reads overlap. Methylation probability threshold of 0.75 was used. **g,** Aggregate browser traces comparing DiMeLo-seq signal to ChIP-seq and CUT&Tag. BED files used for creating aggregate curves are generated either from `parse_bam` or `plot_browser`. CpG methylation signal is aggregated from the H3K27ac-, H3K27me3-, and H3K4me3-targeted DiMeLo-seq experiments. Methylation probability threshold of 0.8 was used. ATAC-seq and NCBI RefSeq annotations are also shown.

The methylation profile centered at features of interest can be visualized using the `plot_enrichment_profile` function. Here, we show profiles from H3K27ac-, H3K27me3-, and H3K4me3-targeted DiMeLo-seq with aggregate methylation curves from reads centered at ChIP-seq peaks of varying strength (Figure 5d-f) (ENCODE ENCFF218QBO, ENCFF119CAV, ENCFF228TWF).[23] H3K27ac and H3K4me3 have narrow peaks, while H3K27me3 has a broader peak. Signals for all three marks track with ChIP-seq peak strength, indicating concordance between DiMeLo-seq and ChIP-seq in aggregate.

To further analyze the concordance between DiMeLo-seq and other methods for measuring protein-DNA interactions - here ChIP-seq and CUT&Tag - we created aggregate browser tracks across a stretch of chromosome 1 (Figure 5g) (ENCODE ENCFF218QBO, ENCFF119CAV, ENCFF228TWF; GEO GSM5530639, GSM5530673).[23,24] DiMeLo-seq signal for all three histone marks tracks with ChIP-seq and CUT&Tag profiles. These curves were generated using the bed file output from the `plot_browser` function with smoothing in a 100 bp window. DiMeLo-seq also measures endogenous CpG methylation together with protein binding. An aggregate mCpG signal from the three DiMeLo-seq samples is shown, and dips in mCpG are evident where H3K27ac and H3K4me3 signals are highest. H3K27ac and H3K4me3 are both marks of open chromatin and have peaks overlapping accumulations in ATAC-seq signal (ENCODE ENCFF603BJO).[23]

In addition to comparing DiMeLo-seq to other methods, we also evaluated methylation profiles around genomic features where our targets are expected to localize. In particular, both H3K27ac and H3K4me3 are found at transcription start sites (TSSs).[25] Using the `plot_enrichment_profile` function, we created the aggregate methylation and single-molecule methylation plots shown in Figure 6a. As expected, both marks have enrichment at the TSSs, with the highest methylation levels at the TSSs for the genes with highest expression.[25] The periodicity from positions 0 bp to 500-1000 bp with respect to the TSS indicate preferential methylation of linker DNA between strongly positioned nucleosomes downstream from the TSSs for both targets. For genes that are not expressed (quartile 1), no significant enrichment at TSSs is evident.

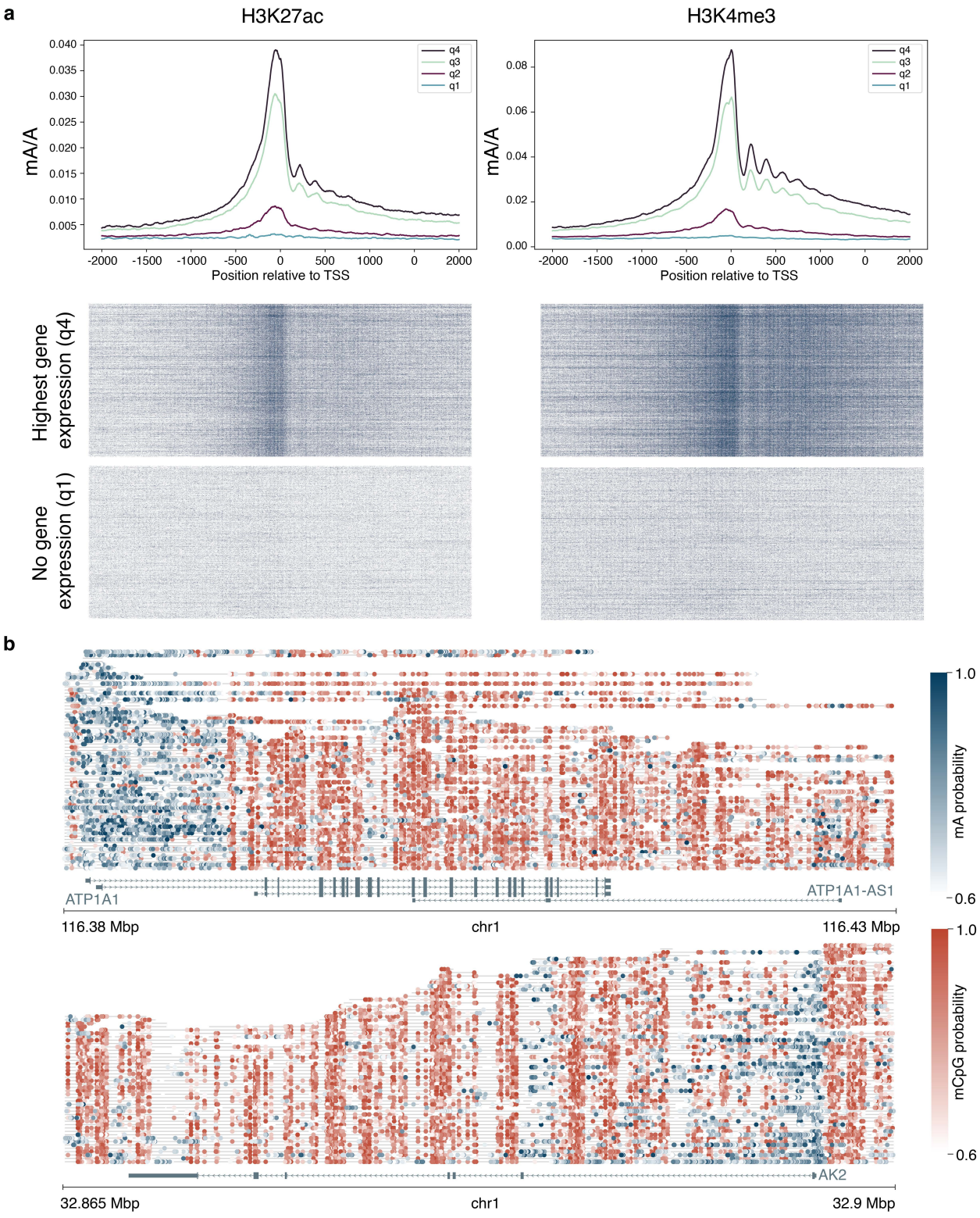


Figure 6. Evaluating protein binding at regions of interest. Both H3K27ac and H3K4me3 are found at transcription start sites (TSS). **a,** Signal from H3K27ac- and H3K4me3-targeted DiMeLo-seq at TSS. Reads overlapping TSS, gated by gene expression level from highest gene expression

(quartile 4 (q4)) to lowest gene expression (quartile 1 (q1)). Aggregate mA/A profiles are shown for all reads spanning these TSSs. Single molecules are shown below with blue representing mA calls for TSS for the highest gene expression (q4) and for no gene expression (q1). Aggregate and single-molecule plots were produced with `plot_enrichment_profile`. Methylation probability threshold of 0.75 was used. **b,** Single-molecule browser plots produced from `plot_browser` from H3K4me3-targeted DiMeLo-seq experiment. Each grey line represents a read, blue circles indicate mA, and red circles indicate mCpG. NCBI RefSeq genes are shown below. Methylation probability threshold of 0.6 was used.

Using the `plot_browser` function, single molecules are shown from H3K4me3-targeted DiMeLo-seq in a window around a few highly expressed genes in GM12878 (Figure 6b). Methylated adenines are enriched around the TSSs for these highly expressed genes ATP1A1 and AK2. Together with mA, the endogenous mCpG can also be analyzed. Here, it is evident that mCpG is depleted in the regions around TSSs where H3K4me3 is enriched, as has been previously reported.[26] Multiple TSSs are spanned by some of the molecules in the region from 116.38 Mbp to 116.43 Mbp on chromosome 1, highlighting DiMeLo-seq's ability to probe multiple binding events on a single molecule.

DiMeLo-seq can be used to target proteins in nuclei isolated not only from cultured cells but also from primary tissue or intact organisms. We mapped H3K9me3 distributions in *D. melanogaster* embryos across the genome and show that averaging methylation signal from single molecules generates profiles consistent with previously published ChIP-seq data (Figure 7).[27] DiMeLo-seq coverage is consistent across the entire *D. melanogaster* genome because DiMeLo-seq's long reads can be mapped in repetitive regions of the genome. We highlight a transition on chr3L where H3K9me3 accumulates and show that the accumulation is evident on single molecules using the `plot_browser` function.

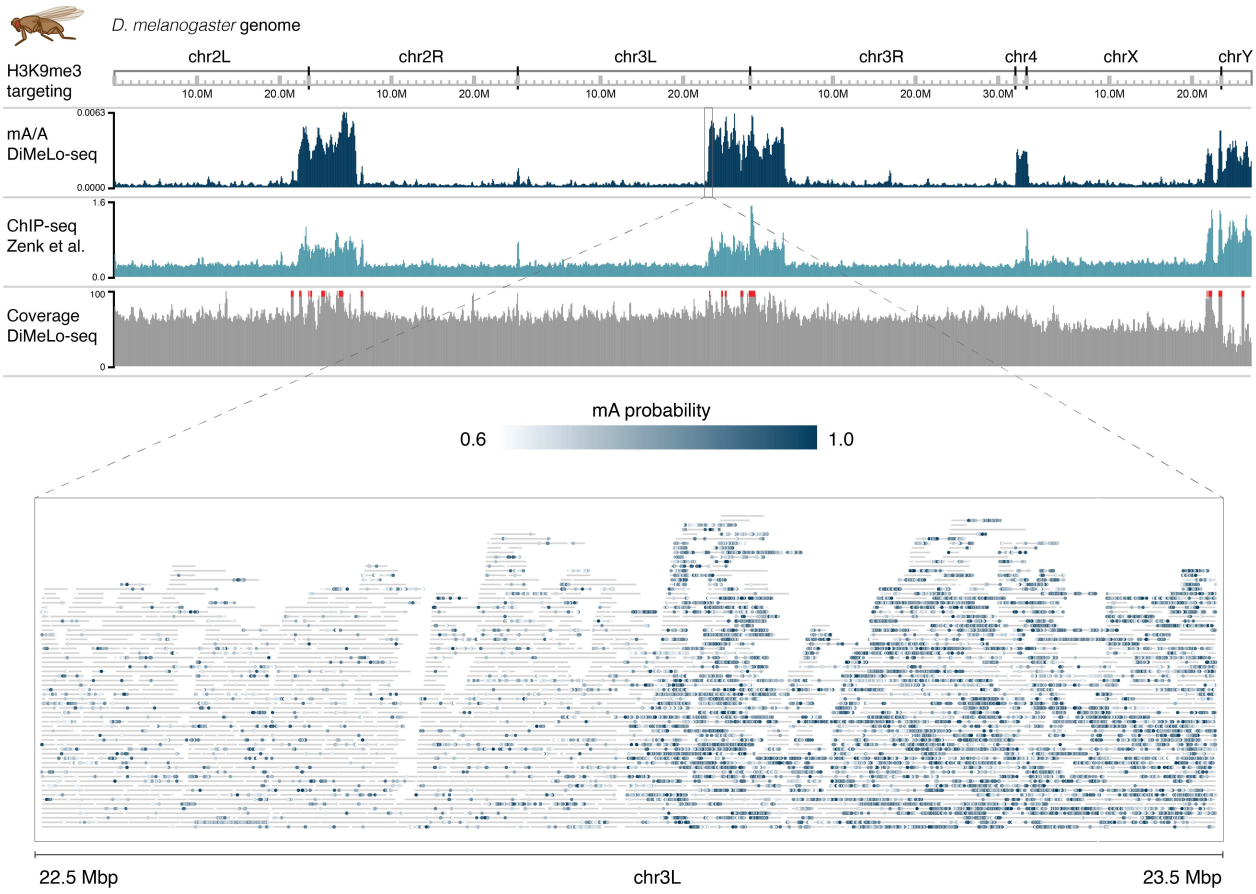


Figure 7. H3K9me3-targeted DiMeLo-seq in *D. melanogaster* embryos. Aggregate mA/A across the entire *D. melanogaster* genome from a DiMeLo-seq experiment targeting H3K9me3 is shown in dark blue. H3K9me3 ChIP-seq data in *D. melanogaster* embryos is shown in light blue.[27] Coverage from the DiMeLo-seq experiment is shown in grey. A region on chr3L where a transition from H3K9me3 depletion to H3K9me3 enrichment is highlighted with a single-molecule browser plot generated from `plot_browser`. Grey lines indicate reads and blue dots indicate mA calls with intensity colored by probability of methylation. An alignment length filter of 10 kb was applied. Methylation probability threshold of 0.6 was used.

The DiMeLo-seq protocol described here enables profiling of protein-DNA interactions in repetitive regions of the genome, makes phasing easier for determining haplotype-specific interactions,[26] detects joint binding events on single molecules of DNA, and captures protein binding together with endogenous CpG methylation. Performance varies by protein target, antibody quality, and chromatin environment; therefore, methylation sensitivity and specificity must be evaluated for each new target. The *dimelo* software package provides tools for quality control and data exploration for the multimodal datasets that DiMeLo-seq produces.

Data availability

All sequencing data are available by request and will be made available on SRA and GEO upon publication. All raw fast5 sequencing data from the accompanying Altemose et al. manuscript are available in the Sequence Read Archive (SRA) under BioProject accession PRJNA752170. H3K27ac ChIP-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF218QBO (https://www.encodeproject.org/files/ENCFF218QBO/). H3K27me3 ChIP-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF119CAV (https://www.encodeproject.org/files/ENCFF119CAV/). H3K4me3 ChIP-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF228TWF (https://www.encodeproject.org/files/ENCFF228TWF/). H3K27ac CUT&Tag data in GM12878 available on Gene Expression Omnibus (GEO) under accession GSM5530639 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM5530639). H3K27me3 CUT&Tag data in GM12878 available on GEO under accession GSM5530673 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM5530673).
ATAC-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF603BJO (https://www.encodeproject.org/files/ENCFF603BJO/). Transcription start site and gene annotations from NCBI RefSeq downloaded from UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgTrackUi?g=refSeqComposite&db=hg38). RNA-seq data in GM12878 available from ENCODE Project Consortium under accession ENCFF978HIY (https://www.encodeproject.org/files/ENCFF978HIY/). *D. melanogaster* H3K9me3 ChIP-seq data available on GEO under accession GSE140539 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140539). File GSE140539_H3K9me3_sorted_deepnorm_log2_smooth.bw was used.

Code availability

The *dimelo* Python package for analysis of DiMeLo-seq data is available on Github: https://github.com/streetslab/dimelo

Author contributions

AM, NA, and AS designed the study. AM, NA, and LDB performed the experiments. AM, RM, and JM developed *dimelo* software package. AM and NA analyzed and interpreted the data. AM, NA, and RM made the figures. AM wrote the manuscript, with input from NA, RM, JM, LDB, KS, GK, AFS, and AS. AS and NA supervised the study.

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Competing interests

N.A., A.M., K.S., A.F.S. and A.S. are co-inventors on a patent application related to this work. The remaining authors declare no competing interests.

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Supplementary Methods

Additional protocol and material availability

DiMeLo-seq: https://doi.org/10.17504/protocols.io.b2u8qezw; pA–Hia5 protein purification: https://doi.org/10.17504/protocols.io.bv82n9ye; AlphaHOR-RES: https://doi.org/10.17504/protocols.io.bv9vn966. Plasmids are available on Addgene: pA–Hia5 expression plasmid (pET-pA–Hia5; Addgene, 174372) and pAG–Hia5 expression plasmid (pET-pAG–Hia5; Addgene, 174373).

Cell culture

GM12878 cells (GM12878, Coriell Institute; mycoplasma tested) were maintained in RPMI-1640 with L-glutamine (Gibco, 11875093) supplemented with 15% FBS (VWR 89510-186) and 1% penicillin–streptomycin (Gibco, 15070063) at 37 °C in 5% CO2.

Antibody information

Antibodies used in this study were: (1) Histone H3K27ac antibody (pAb) (Active Motif 39133), (2) Histone H3K27me3 antibody (pAb) (Active Motif 39055), (3) Histone H3K4me3 antibody (pAb) (Active Motif 39916), (4) Histone H3K9me3 antibody (pAb) (Active Motif 39062).

DiMeLo-seq in GM12878 cells

For each target, 3.24 M cells from fresh culture were input to DiMeLo-seq. Antibody dilutions were all 1:50. DNA extraction was performed using the Monarch Genomic DNA Purification Kit (NEB T3010S).

DiMeLo-seq with D. melanogaster *embryos*

Timed embryo collections for downstream DiMeLo-seq

Approximately 200-400 OregonR flies were maintained on standard molasses medium before transfer to embryo collection cages with apple-juice plates with yeast paste. A heterogeneous mixture of embryos were collected overnight from 2-4 cages and pooled together. Embryos were rinsed from the apple-juice plates with DI water and collected in a mesh sieve, the chorion was removed by soaking in 50% bleach for 90 seconds, and then rinsed with water to remove the bleach. Embryos were transferred to a 1.5 mL eppendorf tube, allowed to settle, and the water was replaced with 1 mL Embryo Storage Buffer. Embryos were frozen in a Mr. Frosty isopropanol bath at -80ºC overnight, then stored at -80ºC. Nuclei were prepped for DiMeLo-seq by thawing the embryos at room temperature, removing the storage buffer and replacing it with

1mL of 1xPBS. Embryos were transferred to a 1 mL glass Dounce homogenizer and lysed with 10-15 strokes of a loose-fitting pestle. Nuclei were gently pelleted at 600xg for 3 minutes at 4ºC, the supernatant was removed, and the pellet was resuspended in Dig-wash buffer for downstream DiMeLo-seq. DNA extraction was performed using the Monarch HMW DNA Extraction Kit (NEB T3050L) with 2000 rpm at lysis.

Materials
Apple juice plates
Yeast paste
Oregon R flies (young) ~ 200 per bottle
Bleach
DI water

Buffers:
Embryo Storage Buffer: 80% Schneider's S2 media (Thermo 21720024), 10% FBS, 10% DMSO
1X PBS
50% bleach solution

Equipment
Small embryo sieve
Paintbrush
Squirt bottle (with DI water)
1 mL pipette and tips
Mr. Frosty isopropanol freezing bath
1.5 mL Eppendorf tubes

Library preparation and sequencing

All library preparation was performed using ON SQK-LSK110 with the standard protocol's bead-based cleanup protocol. Targets in GM12878 were sequenced on PromethION with R9.4.1 flow cells (ON FLO-PRO002). *D. melanogaster* embryo experiments were sequenced on MinION with R9.4.1 flow cells (ON FLO-MIN106D).